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# Neutrophil gelatinase-associated lipocalin and interleukin-10 regulate intramacrophage *Chlamydia pneumoniae* replication by modulating intracellular iron homeostasis

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ABSTRACT

Neutrophil gelatinase-associated lipocalin (NGAL/Lipocalin-2/Lcn-2) is a 25 kDa protein which is involved in host defence against certain Gram negative bacteria upon binding of iron loaded bacterial siderophores thereby limiting the availability of this essential nutrient to bacteria resulting in inhibition of their growth and pathogenicity. As iron is important for the growth of the intracellular bacterium *Chlamydia pneumoniae* we questioned whether Lcn-2 affects the course of this infection. We employed primary peritoneal macrophages obtained from wildtype and Lcn-2 −/− mice and RAW 264.7 cells which were infected with *C. pneumoniae*. In addition, we studied *C. pneumoniae* multiplication *in vivo* in mice receiving diets with varying iron contents. We analyzed *C. pneumoniae* numbers by immunohistochemistry and RT-PCR and studied the expression of iron metabolism and cytokine genes by RT-PCR, Western blot or ELISA.

Infection with *Chlamydiae ex vivo* and *in vivo* revealed a significantly higher bacterial growth in peritoneal macrophages of Lcn-2 −/− than of wildtype mice. These differences were significantly more pronounced upon iron challenge, which stimulated bacterial growth. Accordingly, treatment with an anti-Lnc-2 antibody increased whereas addition of recombinant Lcn-2 reduced bacterial growth in infected macrophages. When investigating the underlying mechanisms we observed partly different expression of several iron metabolism genes between Lcn-2 +/+ and Lcn-2 −/− macrophages and most strikingly an increased formation of the anti-inflammatory cytokine IL-10 by Lcn-2 −/− macrophages. Upon treatment with an anti-IL10 antibody we experienced a significant increase of *Chlamydial* growth within Lcn-2 −/− macrophages along with a reduction of the major iron storage protein ferritin. Herein we provide first time evidence that Lcn-2 is involved in host defence against *Chlamydia* presumably by limiting the availability of iron to the pathogen. In the absence of Lcn-2, increased formation of IL-10 exerts protective effects by increasing the intracellular formation of ferritin, thereby reducing the access of iron for bacteria.



## Introduction

Iron availability is of central importance at the host–pathogen interface (Weinberg 1999; Nairz et al. 2010; Schaible and Kaufmann 2004). On the one hand, most microbes require a sufficient supply of iron which they need for their proliferation and pathogenicity, and many microbial genes are thus designated to ensure the delivery of this metal for microbes (Schaible and Kaufmann 2004; Nairz et al. 2010; Schrettl et al. 2004; Velayudhan et al. 2007). On the other hand, iron exerts subtle effects on anti-microbial immune defence. First, iron has a negative regulatory effect on the efficacy of interferon-gamma (IFN-γ) driven effector pathways of macrophages. Thereby, excess iron inhibits the formation of classical host responses such as tumor necrosis factor-alpha (TNF-α), interleukin-6 (IL-6) or nitric oxide (NO) formation which results in an impaired control of infections with intracellular pathogens such as *Salmonella* or *Mycobacterium tuberculosis* (Weiss et al. 1994; Olakanmi et al. 2002; Oexle et al. 2003; Appelberg 2006; Nairz et al. 2007). In addition, iron differently affects the proliferation and differentiation of lymphocyte subsets either directly or via modulation of cytokine activities taking part in immune cell differentiation

*Abbreviations:* aIL-10, anti-interleukin-10; *C. pneumoniae*, Cpn, *Chlamydia pneumoniae*; DMT-1, divalent metal transporter-1; Fe, iron; Fpn1, ferroportin; hamp, hepcidin; HPRT, hypoxanthine guanine phosphoribosyl transferase; Lcn-2, Lipocalin-2; IL-10, interleukin-10; TfR, transferrin receptor; Lcn-2 +/+, wt, wildtype.

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(Recalcati et al. 2010; Weiss 2002). Third, minute amounts of iron are also needed for the catalytic formation of reactive oxygen radicals as part of the anti-microbial effector pathways of macrophages (Rosen et al. 1995). Thus, the control over iron homeostasis may decide about the fate of an infection.

The control over iron homeostasis is thus vital to the course of infection and withholding the metal from microbes has been proven to be an efficient strategy for infection control (Schaible and Kaufmann 2004; Weinberg 2000). Accordingly, it has been demonstrated that during infection or inflammation significant regulatory changes of macrophage iron homeostasis occur which may be further modified as a consequence of pathogen localization (intra-versus extracellular) or its needs for iron (Wessling-Resnick 2010; Weiss 2002; Nairz et al. 2010).

Macrophages can acquire iron by multiple pathways including uptake of transferrin bound iron via transferrin receptor mediated endocytosis, acquisition of molecular iron by the activity of divalent metal transporter-1 (DMT-1) or via erythrophagocytosis, a physiological mechanism to reutilize iron from senescent erythrocytes (Hentze et al. 2010; Wessling-Resnick 2010; Delaby et al. 2005; Knutson et al. 2005; Weiss 2009). Iron is then either stored upon incorporation into ferritin or released from the cell, a pathway which is maintained by a single transcellular transport protein termed ferroportin (Fpn1) (Hentze et al. 2010).

The expression of these iron transport proteins is modified by the action of different cytokines, acute phase proteins and microbial products. While IL-1, IL-6, TNF-α, IFN-γ but also the "anti-inflammatory" cytokines IL-4, IL-10 and IL-13 increase uptake of iron into isolated macrophages, LPS, IFN-γ and the master regulator of iron homeostasis, hepcidin, block its export by inhibiting ferroportin expression transcriptionally and post-translationally, respectively (Wessling-Resnick 2010; Weiss 2009; Ludwiczek et al. 2003; Hentze et al. 2010). Thus, iron is retained within macrophages and stored within ferritin whose expression is strongly induced by inflammatory cytokines. These events lead to reduction of circulating iron levels which should limit the availability of iron for extracellular pathogens but which also results in the development of anemia of inflammation (Wessling-Resnick 2010; Weinberg 1999; Weiss 2009). Interestingly, when macrophages are targeted with the intracellular pathogen *Salmonella typhimurium* they induce an opposite effect by reducing iron uptake and increasing iron export via stimulation of ferroportin expression thereby limiting iron availability for *Salmonella* and increasing anti-microbial immune effector pathways (Nairz et al. 2007, 2008). In a line with this, the modulation of ferroportin functionality was associated with altered proliferation kinetics of intracellular pathogens such as *Salmonella*, *Chlamydia trachomatis* or *Legionella pneumophila* (Chlosta et al. 2006; Paradkar et al. 2008; Nairz et al. 2007).

*Chlamydia* or *Chlamydophila* are obligatory intracellular bacteria, and the subspecies *Chlamydia pneumoniae* has been associated with respiratory tract infections but also with the pathogenesis of atherosclerosis (Campbell and Kuo 2004; Belland et al. 2004; Rosenfeld and Campbell 2011). *C. pneumoniae* perform a unique developmental cycle. Upon endocytosis the infectious elementary bodies differentiate to non-infectious though metabolically active reticular bodies utilizing cellular nutrients and influencing cellular metabolism. *C. pneumoniae* is capable to infect a variety of cells of the immune system but has been also detected in smooth muscle cells, vascular endothelial cells and in atherosclerotic plaques (Bachmaier and Penninger 2005; Lajunen et al. 2008). Earlier studies established that iron is essential for propagation and pathogenicity of *C. pneumoniae* infection (Paradkar et al. 2008; Bellmann-Weiler et al. 2010), however, the detailed abilities of iron acquisition system by the pathogen remained largely elusive so far.

As a major pathway for iron acquisition Gram negative bacteria such as *S. typhimurium*, *Escherichia coli* or *Klebsiella pneumoniae* produce and release siderophores. As a defence against this microbial siderophore system neutrophils and macrophages produce a 25 kDa protein called neutrophil gelatinase associated lipocalin (NGAL) or Lipocalin-2 (Lcn-2). Lcn-2 exerts important bacteriostatic activity toward *Salmonella*, *E. coli* or *Klebsiella* spp. by capturing iron loaded bacterial siderophores, such as enterobactin, thereby leading to an improved control of infections in mice (Flo et al. 2004; Berger et al. 2006; Nairz et al. 2009). Although *Chlamydia* are not known to produce siderophores, Lcn-2 can modulate intracellular iron availability independently of bacterial siderophore binding via its putative interaction with recently identified mammalian siderophores (Bao et al. 2010; Devireddy et al. 2010), thereby tailoring the access of iron for such microbes (Nairz et al. 2009) and/or affecting iron sensitive immune effector pathways (Weiss 2002). As no data were available so far on a putative function of Lcn-2 in *Chlamydia* infection we herein investigated the role of this peptide on the intramacrophage proliferation of *Chlamydia*, along with its effects on macrophage iron homeostasis and innate immune effector pathways.

## Materials and methods

### *Animals, cell culture and infection with C. pneumoniae*

Female C57BL/6 mice (age 8–12 weeks) of the two genotypes Lcn-2 +/+ (wildtype/wt) and Lcn-2 −/−, respectively, were kept according to the guidelines of the Medical University of Innsbruck (Innsbruck, Austria) and the Austrian Ministry for Science and Education (based on the Austrian Animal Testing Act of 1989; BMWF-66.011/77-II/10b/2008) in the animal quarter at the Medical University of Innsbruck. Mice were fed a standard rodent diet. Thioglycolate-elicited primary peritoneal macrophages were harvested from mice and cultured in DMEM containing 5% fetal calf serum (FCS; both purchased from PAA Laboratories GmbH, Vienna, Austria), 100 U/mL penicillin, 0.1 mg/mL streptomycin, and 2 mM L-glutamine (all from PAA Laboratories GmbH).

The *C. pneumoniae* strain CV-6 (kindly provided by Jan Rupp, Institute of Medical Microbiology and Hygiene, University of Lübeck, Germany) was cultivated and propagated as described earlier (Bellmann-Weiler et al. 2010). For experiments, $1.5 \times 10^6$ cells were seeded in 6-well plates and infected with purified *C. pneumoniae* at a multiplicity of infection of 10 as described (Bellmann-Weiler et al. 2010).

Upon infection primary macrophages were stimulated with either 50 μM ferrous sulfate ($FeSO_4$), 10 μg/mL of a blocking rat monoclonal anti-mouse interleukin-10 antibody (aIL-10) (BioLegend, San Diego, CA, USA) or both, or a purified rat IgG1 antibody (BioLegend, San Diego, CA, USA) which served as a isotype control. In parallel experiments were performed upon stimulation of cells with 10 ng/mL and 50 ng/mL of recombinant mouse interleukin-10 (BioLegend, San Diego, CA, USA), respectively.

For *in vivo* experiments mice of both genotypes were fed either an iron enriched (25 g Fe/kg; Altromin GmbH, Lage, Germany), an iron deficient (5.2 mg Fe/kg; Altromin GmbH, Lage, Germany) or standard diet (180 mg Fe/kg; Altromin GmbH, Lage, Germany) for 21 days. Thereafter mice were injected with thioglycolate and after three days *C. pneumoniae* ($1.5 \times 10^7$ IFU/mouse, suspended in 200 μL PBS) was injected intraperitoneally in mice of all dietary groups. Animals were sacrificed 9–12 h later and peritoneal macrophages were harvested. The extent of infection was quantified by microscopic examination of macrophages and RT PCR for *C. pneumoniae* DNA copies as described below. The concentration of interleukin-10 was measured in the peritoneal fluid by means of ELISA (BD Pharmingen, Biosciences, San Jose, CA).

For microscopic examination of infection cells were fixed in methanol and stained with fluorescein isothiocyanate-conjugated anti-*Chlamydia* lipopolysaccharide monoclonal antibody (Oxoid Ltd., Ely, UK) on coverslips. *Chlamydial* inclusion bodies within cells were counted by fluorescence microscopy at a 500× magnification using a Scope A1 microscope (Zeiss).

For experiments with RAW 264.7 cells $1 \times 10^6$ cells were seeded in 6-well plates and infected with purified *C. pneumoniae* at a multiplicity of infection of 2 as described (Bellmann-Weiler et al. 2010). After 1 h, cells were stimulated with 10 μg/mL of purified anti-mouse IL-10 antibody (BioLegend, San Diego, CA), 10 ng/mL of recombinant mouse IL-10 (BioLegend, San Diego, CA), 25 μg/mL of monoclonal anti-mouse Lipocalin-2/NGAL antibody (aLcn-2, clone 228418) or 25 ng/mL of recombinant mouse Lcn-2 (apo-Lcn-2, free of iron and microbial siderophores, both from R&D Systems, Biomedica, Vienna, Austria) respectively. The anti-Lcn-2 antibody blocks the biological activity of Lcn-2 which has been specifically demonstrated in wt and Lcn-2 −/− neutrophils and macrophages infected with the Lcn-2 sensitive bacterium *S. typhimurium* (Nairz et al. 2009; Schroll et al. 2012; Fritsche et al. 2012). After 12 h total RNA and bacterial DNA were isolated as described below.

### *RNA/DNA preparation, reverse transcription, and TaqMan polymerase chain reaction*

Bacterial DNA and mammalian RNA was extracted from cells with the guanidine-thiocyanate/phenol mixture (Peqgold trifast; Peqlab, Polling, Austria) and prepared according to the manufacturer's protocol. Reverse transcription was performed with 1 μg of total RNA, random hexamer primers, dNTPs, and Moloney murine leukemia virus reverse transcriptase (all obtained from Invitrogen, Vienna, Austria) in 1× reverse transcription buffer for 1.5 h at 37 °C and PCRs of genes of interest were carried out as described (Ludwiczek et al. 2007). The results were related to the cDNA expression of the house keeping gene hypoxanthine guanine phosphoribosyl transferase/HPRT. Results are expressed as relative cDNA abundance as described (Ludwiczek et al. 2007).

For quantification of *Chlamydia* numbers we performed a 23S-based *Chlamydiaceae* family specific real-time PCR as described (Sostaric-Zuckermann et al. 2011; Mannonen et al. 2011). All TaqMan probes and primers were obtained from Microsynth (Microsynth AG, Balgach, Switzerland). Amplification conditions for *Chlamydia* were 95 °C for 2 min, 40 cycles of 95 °C for 3 s, 62 °C for 6 s.

The following primers and probes were used for the experiments described: muTfR1: 5′-CGCTTTGGGTGCTGGTG-3′, 5′-GGG CAAGTTTCAACAGAAGACC-3′, 5′-CCCACACTGGACTTCGCCGCA-3′; muDmt-1: 5′-GGACTGTGGACGCTCGGTAA-3′, 5′-AATGTTGCCACCG CTGGT-3′, 5′-CATCTCGAAAGTCCTGCTGAGCGAAGA-3′; muH-ferritin: 5′-GCGAGGTGGCCGAATCT-3′, 5′-CAGCCCGCTCTCCCAGT-3′, 5′-CCTGCAGGATATAAAGAAACCAGACCGTGA-3′; muFpn1: 5′-CTACCATTAGAAGGATTGACCAGCT-3′, 5′-CAAATGTCATAATCTG GCCGA-3′, 5′-CAACATCCTGGCCCCCATGGC-3′; muHamp: 5′-GGCAGACATTGCGATACCAAT-3′, 5′-TGCAACAGATACCACACTGGG AA-3′, 5′-CCAACTTCCCCATCTGCATCTTCTGC-3′; h*Chlamydia* ArgR: 5′-ACCAGGAACCGTACGAATTACAA-3′, 5′-GACCACGACCC GTCATTTG-3′, 5′-AAGAGAGGCGTTATGGCGAATAGAGAGCA-3′; muHprt: 5′-GACCGGTCCCGTCATGC-3′, 5′-TCATAACCTGGTTCAT CATCGC-3′, 5′-ACCCGCAGTCCCAGCGTCGTC-3′.

### *Determination of cytokine levels*

Concentrations of TNF-α (R&D Systems, Biomedica, Vienna, Austria), IL-6 and IL-10 (both from BD Pharmingen, Biosciences, San Jose, CA, USA) in supernatants were determined by specific ELISA kits (BD Pharmingen, Biosciences, San Jose, CA, USA) following the manufacturer's instructions.

### *Western blot analysis*

Protein extracts from peritoneal macrophages were prepared as described (Ludwiczek et al. 2007) and 30 μg of protein was run on a 10% sodium dodecyl sulfate polyacrylamide gel and blotted onto nylon membranes (Hybond P; Little Chalfont, Buckinghamshire, UK). After blocking, the membrane was incubated with either a rabbit anti-human ferritin antibody (2.5 μg/mL; Zymed, San Francisco, CA, USA), or a rabbit anti-actin antibody (2 μg/mL; Sigma–Aldrich, Vienna, Austria), which was used to prove equal protein loading onto the gel.

### *Statistical analysis*

Statistical analysis was performed using the Statistics package for the Social Science software package (SPSS, version 15; SAS Institute). Calculations for statistical differences between the various groups were performed either by using the analysis of variance technique with Bonferroni correction for multiple variables, the two-sided Students' *t*-test, or the Mann–Whitney-*U* test depending on the nature and distribution of the basic data. Data were tested for normality by the Kolmogorov–Smirnov test and are given as arithmetic mean ± SEM. *p*-Values of <0.05 were assumed as statistically significant differences.

## Results

### *Effect of Lcn-2 expression on infection with C. pneumoniae in peritoneal macrophages*

As iron is an essential nutrient for the intracellular bacterium *C. pneumoniae* and because Lcn-2 has previously been shown to affect cellular iron homeostasis in macrophages (Devireddy et al. 2005) and the course of infection with the Gram-negative bacterium *S. typhimurium* independently of its siderophore scavenging activity (Nairz et al. 2009) we first studied for a putative effect of Lcn-2 on *C. pneumoniae* infection.

Infection of peritoneal macrophages from C57BL/6/wt/Lcn-2 +/+ and Lcn-2 −/− mice with *C. pneumoniae* indicated significantly higher pathogen numbers as evidenced by immunofluorescence and PCR in Lcn-2 −/− macrophages than in wt cells ($p = 0.002$, Fig. 1A and B). The addition of ferrous iron to the culture medium 1 h after infection resulted in a 3.4-fold and 4-fold increase in the number of pathogens in wt and Lcn-2 −/− macrophages respectively. However, the difference in pathogen numbers between wt and Lcn-2 −/− cells further increased (Fig. 1A and B).

To see whether this effect is directly related to the presence/absence of Lcn-2 or rather due to changes in macrophage functionality as a result of Lcn-2 deletion (Schroll et al. 2012) we employed the murine macrophage cell line RAW 264.7 and infected these cells with *C. pneumoniae*. Addition of an anti-Lcn-2 antibody resulted in a significant increase (3-fold) in bacterial numbers as compared to isotype treated control macrophages whereas the supplementation of recombinant Lcn-2 decreased intramacrophage bacterial counts in relation to the control as quantified by RT-PCR (Fig. 1C).

### *Effect of Lcn-2 on iron metabolism in C. pneumoniae infected macrophages*

Having established that the presence of Lcn-2 significantly affects the course of infection with the intracellular bacterium *C.*

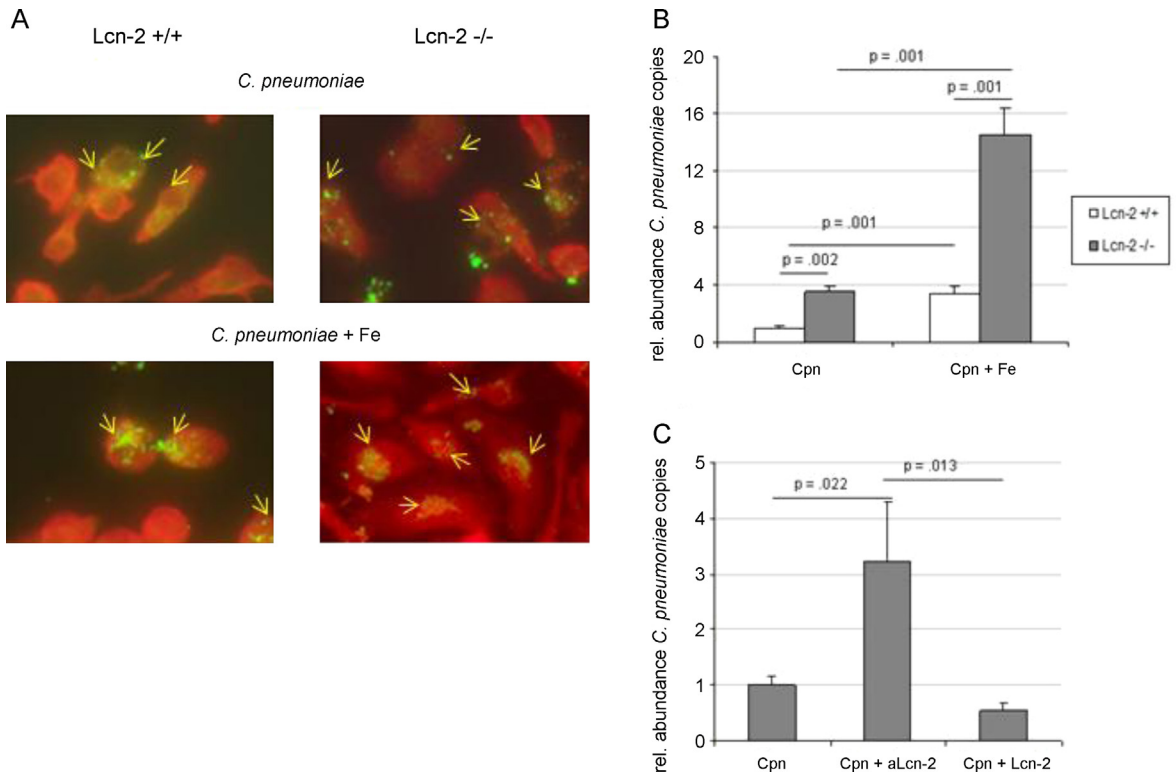


**Fig. 1.** Effect of Lcn-2 expression on *C. pneumoniae* multiplication in macrophages. (A and B) Primary peritoneal macrophages of wt (Lcn-2 +/+) and Lcn-2 −/− mice were infected with *C. pneumoniae* at a MOI of 10 and thereafter stimulated with iron-sulfate (Fe; 50 μM). After 12 h pathogen numbers in cells were quantified by RT-PCR and immunofluorescence using a Scope A1 microscope (Zeiss). Representative slides for control and iron-stimulated macrophages are depicted (500× magnification), fluorescein isothiocyanate-marked *C. pneumoniae* inclusions are indicated by arrows (A). The number of *C. pneumoniae* copies relative to infected wt cells depicted as means ± SEM (B) is shown. (C) RAW264.7 cells were infected with *C. pneumoniae* at a MOI of 2 and anti-Lcn-2 antibody (25 μg/mL) or recombinant Lcn-2 (25 ng/mL) were added 1 h after infection. *C. pneumoniae* numbers were quantified by RT-PCR. Data are shown for 3–5 independent experiments performed in duplicate. Calculations for statistical differences between various groups were calculated by means of Student's *t*-test.

*pneumoniae* we next tried to elucidate the mechanisms underlying this observation.

As we also noted that iron supplementation increased pathogen numbers more significantly in Lcn-2 −/− than wt macrophages we first studied iron homeostasis in these cells. While baseline mRNA expression of the iron uptake gene TfR was significantly ($p = 0.007$) higher in wt than in Lcn-2 −/− macrophages, likewise indicating reduced intracellular iron availability in wt cells (Hentze et al. 2010), these differences completely disappeared upon infection resulting in a reduction of TfR mRNA expression (Fig. 2A). When studying the expression of DMT-1 which accounts for non-transferrin mediated iron uptake into macrophages (Ludwiczek et al. 2003) we found that although DMT-1 mRNA expression was twice higher upon infection with *C. pneumoniae*, the induction was four times more prominent in Lcn-2 −/− macrophages than in wt cells (Fig. 2B).

As *C. pneumoniae* infections differently affected these two iron uptake systems we then investigated putative differences in the expression of ferritin, the gene for intracellular iron storage, and ferroportin, the only known cellular iron exporter.

The expression of H-ferritin mRNA was significantly induced in wt ($p = 0.001$) and Lcn-2 −/− ($p = 0.03$) macrophages upon *C. pneumoniae* infection, however, with insignificantly higher expression in Lcn-2 −/− cells (Fig. 2C). Interestingly, while H-ferritin mRNA expression could be significantly stimulated in *C. pneumoniae* infected peritoneal wt macrophages upon the addition of iron ($p = 0.001$) this was not the case in Lcn-2 −/− cells.

When we studied the mRNA levels of the iron exporter ferroportin we found it highly expressed at baseline and better inducible upon exogenous iron administration in Lcn-2 −/− ($p = 0.002$) than in wt ($p = 0.001$) cells (Fig. 2D), which contrasted the comparative effects observed for H-ferritin mRNA expression (Fig. 2C).

Most interestingly, *C. pneumoniae* infection resulted in a five-fold increase of ferroportin mRNA expression in wt cells, whereas in Lcn-2 −/− macrophages ferroportin mRNA levels decreased by approximately 50% as compared to unstimulated cells and were significantly lower than in infected wt macrophages. In addition, administration of iron during infection stimulated ferroportin mRNA expression in Lcn-2 −/− but not in wt macrophages (Fig. 2D).

### *Effect of Lcn-2 on cytokine expression in infected primary mouse macrophages*

As Lcn-2 alters iron homeostasis and because intracellular iron levels have subtle effects on anti-microbial immune effector pathways of macrophages (Wessling-Resnick 2010; Weiss et al. 1992; Weiss 2002; Oexle et al. 2003) we investigated innate immune responses in *C. pneumoniae* infected wt and Lcn-2 −/− macrophages. Determination of TNF-α, IL-6 and IL-12 in tissue culture supernatants revealed an induction of these cytokines upon *C. pneumoniae* infection, however, with no difference between the two genotypes. In contrast, the expression of IL-10 was found to be significantly higher in Lcn-2 −/− than in wt macrophages (Fig. 3), with most pronounced differences ($p = 0.04$ and $p = 0.03$) at 6 and 12 h (Suppl. Fig. 1). As IL-10 is a cytokine with well described anti-inflammatory properties which are considered to be of disadvantage in bacterial infections (Saraiva and O'Garra 2010) and because IL-10 has been shown to regulate macrophage iron homeostasis (Tilg et al. 2002; Ludwiczek et al. 2003), we questioned whether the increase of IL-10 in Lcn-2 −/− mice may underlie the

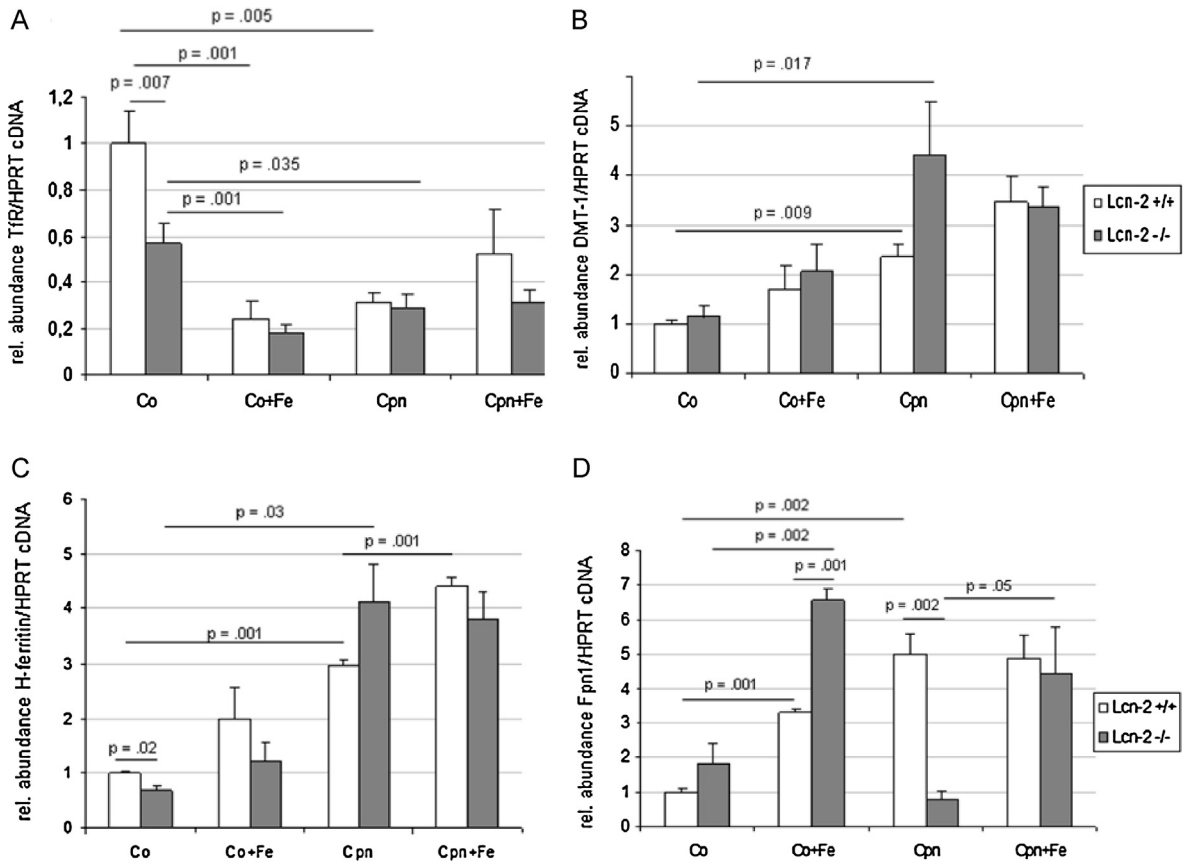


**Fig. 2.** Effects of Lcn-2 on the expression of iron metabolism genes in *Chlamydia* infected macrophages. Wildtype (wt, Lcn-2 +/+; white bars) and Lcn-2 −/− (dark gray bars) macrophages were infected as detailed in the legend to Fig. 1. Transferrin receptor (TfR) (A), divalent metal transporter-1 (DMT-1) (B), H-ferritin (C) and ferroportin (Fpn1) (D) mRNA levels were determined by quantitative RT-PCR. Values were corrected for the amount of the house keeping gene hypoxanthine guanine phosphoribosyl transferase (HPRT). Results are shown as relative abundance of the respective cDNAs relative to unstimulated control (Co) cells. Data are expressed as mean ± SEM for five independent experiments performed in duplicates. *p*-Values < 0.05 indicated significant differences.

changes in iron gene expression and/or the increased proliferation of *C. pneumoniae* within primary macrophages obtained from Lcn-2 −/− mice.

Supplementary data associated with this article can be found, in the online version, at http://dx.doi.org/10.1016/j.imbio.2012.11.004.

We therefore analyzed the course of infection in the presence of a blocking anti-interleukin-10 antibody (aIL-10). As evidenced above (Fig. 1) *C. pneumoniae* proliferation was significantly higher

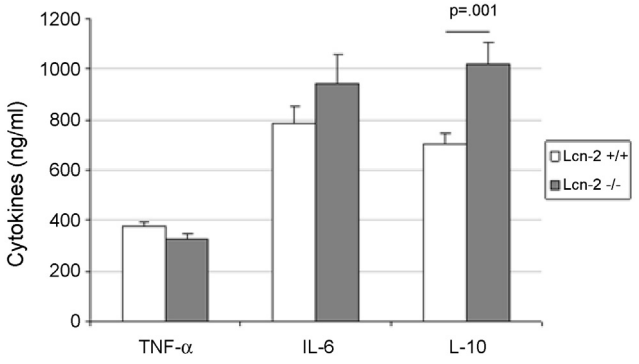


**Fig. 3.** Effects of Lcn-2 on cytokine expression in *Chlamydia* infected macrophages. Primary peritoneal macrophages of both genotypes were infected and treated as described in the legend to Fig. 1. Concentrations of IL-6, IL-10 and TNF-α were determined in cell culture supernatants by ELISA. Data are expressed as mean ± SEM for five independent experiments performed in duplicates. *p*-Values < 0.05 indicated significant differences.

in Lcn-2 −/− and paralleled by increased IL-10 levels as compared to wt macrophages. Surprisingly, the addition of aIL-10 lead to significantly increased *C. pneumoniae* proliferation in Lcn-2 −/− macrophages, and this effect could be further promoted by the addition of iron whereas aIL-10 treatment exerted little effects in wt cells (Fig. 4A and B).

However, we could re-confirm the microbial growth promoting effect of aIL-10 in RAW264.7 macrophages infected with *C. pneumoniae* (Fig. 4C). In addition, stimulation of *C. pneumoniae* infected peritoneal macrophages with interleukin-10 (10 ng/mL) resulted in a 2.3-fold reduction in *C. pneumoniae* numbers in wt mice and in a 3.7-fold decrease of *C. pneumoniae* counts in peritoneal macrophages from Lcn-2 −/− mice. As a control peritoneal macrophages were treated with an anti-interleukin isotype antibody (Fig. 4D). Treatment of cells with a higher IL-10 dose (50 ng/mL) did not result in any significant changes of *C. pneumoniae* DNA numbers as compared to treatment with 10 ng/mL IL-10 independent of the underlying Lcn-2 genotype (details not shown).

We then studied whether aIL-10 treatment would affect the expression of iron metabolism genes in *C. pneumoniae* infected macrophages. While addition of aIL-10 to infected wt macrophages increased H-ferritin and DMT-1 mRNA expression, it insignificantly reduced ferroportin mRNA levels (Fig. 5A–C). In contrast, in infected Lcn-2 −/− macrophages administration of aIL-10 resulted in a significant increase of the albeit low ferroportin mRNA expression whereas all other iron genes investigated remained largely unaffected (Fig. 5D). To confirm these effects of aIL-10 we next analyzed ferritin protein levels by means of Western blot analysis

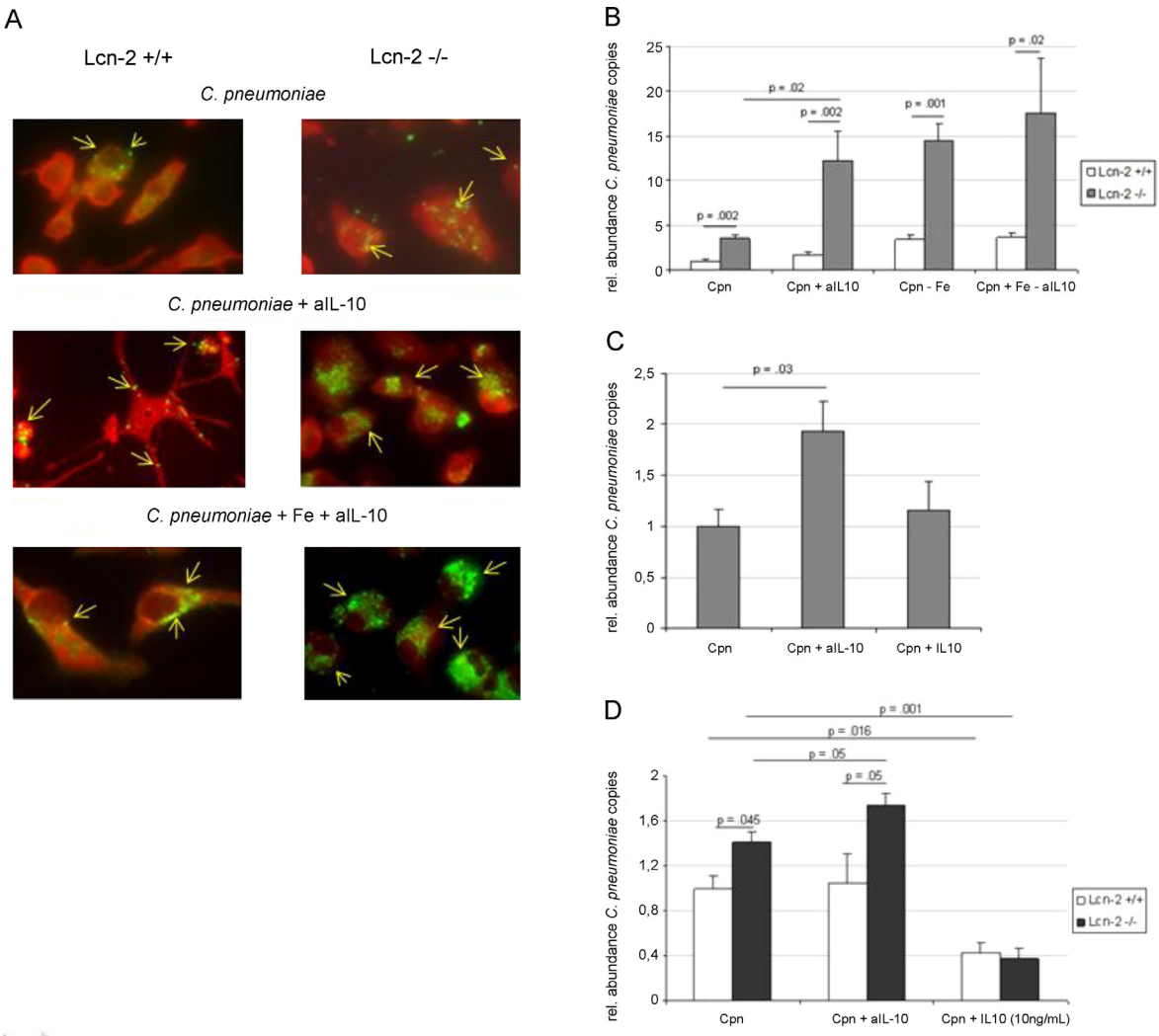


**Fig. 4.** Effects of interleukin-10 neutralization on *C. pneumoniae* multiplication in macrophages. (A and B) Primary peritoneal macrophages from Lcn-2 +/+/wt and Lcn-2 −/− mice were infected with *C. pneumoniae* at a MOI of 10 and thereafter treated with an anti-IL-10 antibody (aIL-10; 10 μg/mL) and/or ferrous sulfate (Fe, 50 μM) and/or an isotype antibody serving as control. After 12 h pathogen numbers in cells were quantified by RT-PCR and immunofluorescence. Representative slides are depicted at a 500× magnification using a Scope A1 microscope (Zeiss), fluorescein isothiocyanate-marked *C. pneumoniae* inclusions are indicated by arrows (A). Expression of *C. pneumoniae* copies are depicted as means ± SEM (B). (C) RAW264.7 cells were infected with *C. pneumoniae* at a MOI of 2 and aIL-10 (10 μg/mL) or recombinant murine IL-10 (IL-10; 10 ng/mL) were added immediately after infection. *C. pneumoniae* numbers were quantified by RT-PCR. Data are shown for 3–5 independent experiments performed in duplicate. *p*-Values < 0.05 indicate statistically significant differences between various groups. (D) Mouse peritoneal macrophages were stimulated with anti-IL-10 antibody or recombinant IL-10 after infection; controls were supplemented with an isotype control antibody or left untreated. No difference was observed concerning pathogen numbers between isotype antibody treated cells and untreated control cells (the latter not shown). *C. pneumoniae* copies were quantified by RT-PCR and are depicted as means ± SEM for three independent experiments performed in triplicates.

and found lower ferritin levels in Lcn-2 −/− primary macrophages when compared to wt cells after treatment with aIL-10 (Fig. 5E). This effect was reversed by additional stimulation with iron. The evaluation of ferroportin protein expression by Western blotting was not feasible due to the lack of a good anti-mouse ferroportin antibody.

However, as the protein expression of ferroportin is controlled by hepcidin (Nemeth et al. 2004) and because macrophages have been shown to produce hepcidin in response to inflammatory stimuli thereby modulating ferroportin expression in an autocrine fashion (Theurl et al. 2008; Peyssonnaux et al. 2006) we studied hepcidin mRNA expression under these conditions.

While comparable mRNA levels of hepcidin were detected in *C. pneumoniae* infected primary macrophages of both genotypes, treatment of Lcn-2 −/− macrophages with aIL-10 resulted in a significant induction of hepcidin expression whereas it remained largely unaffected in wt macrophages (Fig. 5F).

### *Course of C. pneumoniae infection in wt and Lcn-2 −/− mice fed diets with varying iron contents*

To further explore the role of iron, IL-10 and Lcn-2 for *Chlamydia* infection *in vivo* we fed wt and Lcn-2 −/− with an iron enriched, an iron deficient or a standard diet for three weeks. Animals were then infected with *C. pneumoniae* intraperitoneally, and infection of peritoneal macrophages and the levels of IL-10 were quantified in each dietary group. We reconfirmed significantly higher pathogen numbers in Lcn-2 −/− mice when compared to wt mice ($p = 0.01$), and this difference was even more pronounced in animals fed an iron enriched diet (Fig. 6A and B). On the other hand dietary iron restriction resulted in significantly decreased infection rate in Lcn-2 −/− but not in wt mice (Fig. 6A and B). In parallel, we found that the concentrations of IL-10 in the peritoneal cavity was approximately 8-fold higher in Lcn-2 −/− than in wt mice (Fig. 6C) which confirmed our previous results obtained *in vitro* and *ex vivo*. Moreover,

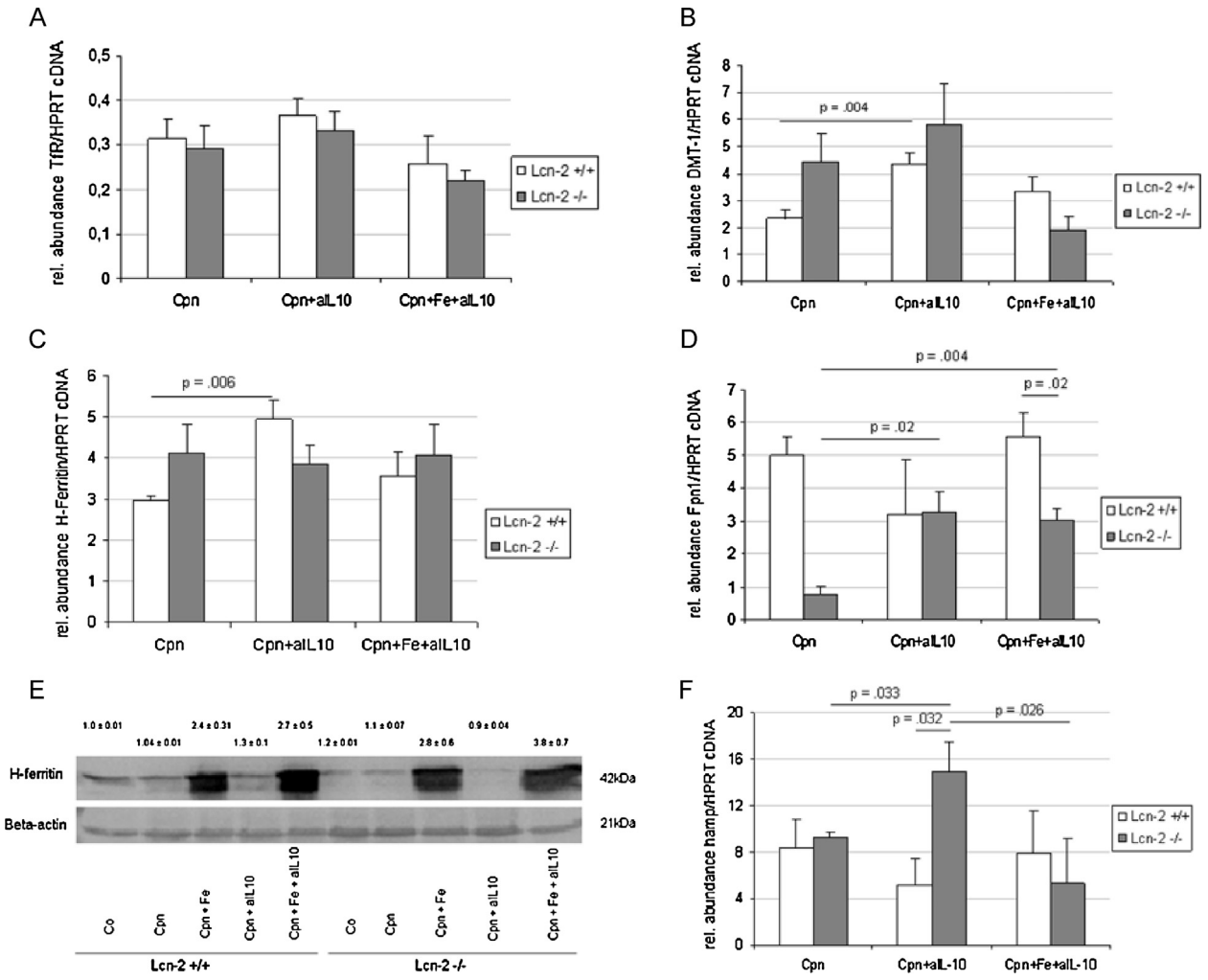


**Fig. 5.** Impact of Lcn-2 and interleukin-10 on expression of iron metabolism genes in *Chlamydia* infected macrophages. (A–D) wt/Lcn-2 +/+ (white bars) and Lcn-2 −/− (dark gray bars) – macrophages were infected with *C. pneumoniae* (Cpn) at a MOI of 10 and stimulated as described in the legend to Fig. 4. Transferrin receptor (TfR) (A), divalent metal transporter-1 (DMT-1) (B), H-ferritin (C), ferroportin (Fpn1) (D) and hepcidin antimicrobial peptide (hamp) mRNA (F) levels were quantified as described in the legend to Fig. 2. Data are expressed as means ± SEM for five independent experiments performed in duplicates. *p*-Values < 0.05 indicated significant differences between the groups. (E) H-ferritin protein expression was determined by means of Western blots after 24 h of infection as detailed in "Materials and methods" section. Equal loading of samples was confirmed by re-probing the membranes with an anti-β-actin antibody (lower panel). One of four representative experiments is shown.

a high dietary iron content resulted in significantly increased IL-10 formation in infected wt and Lcn-2 −/− mice as compared to mice fed an iron balanced diet, whereas dietary iron restriction caused a significant decrease of IL-10 levels in Lcn-2 −/− animals in comparison to mice on standard diet ($p = 0.001$), while no comparable decrease was observed for the albeit low IL-10 formation in wt mice (Fig. 6C).

## Discussion

We herein show for the first time that Lcn-2 plays an important role for host defence against *C. pneumoniae*. The protective role of Lcn-2 has been traced back to the binding of iron loaded bacterial siderophores, such as enterobactin, thereby reducing the availability of the essential nutrient iron to gram negative, siderophore producing bacteria such as *Salmonella*, *E. coli* or *Klebsiella* (Flo et al. 2004; Berger et al. 2006; Nairz et al. 2009). Our findings now extend the role of Lcn-2 beyond that mechanism in showing that Lcn-2 also impairs the proliferation of the intracellular pathogen *C. pneumoniae* for which siderophore synthesis has not been shown so far. Our *in vivo* and *in vitro* data further suggest that this protective effect of Lcn-2 can be traced back to modulation of iron availability for *Chlamydia* which is in accordance with data showing that Lcn-2 expression can affect cellular iron homeostasis (Devireddy et al. 2010; Nairz et al. 2009) which may be due to interaction of Lcn-2 with a recently identified mammalian siderophore (Bao et al. 2010; Devireddy et al. 2010). Moreover, we found that essential genes for iron homeostasis were differently expressed in infected Lcn-2 +/+ and Lcn-2 −/− macrophages. Most strikingly, the expression of ferroportin mRNA was reduced in Lcn-2 −/− peritoneal macrophages infected with *C. pneumoniae* whereas it significantly increased in wt macrophages as compared to non-infected cells of the same genotype. This is of importance because previous studies have indicated a decisive role of ferroportin in *Chlamydia* infection which is based on ferroportin mediated cellular iron egress, thus depriving the pathogen from this nutrient (Dill and Raulston 2007; Paradkar et al. 2008) whereas inhibition of iron export upon hepcidin mediated ferroportin degradation promoted *Chlamydial* proliferation (Paradkar et al. 2008). This is in a line with our observations demonstrating improved infection control in wt macrophages with high expression of ferroportin and poor infection control in Lcn-2 −/− macrophages presenting with decreased ferroportin mRNA levels. However, the pathway underlying this observation remains elusive but appears to be linked to Lcn-2 functionality rather than

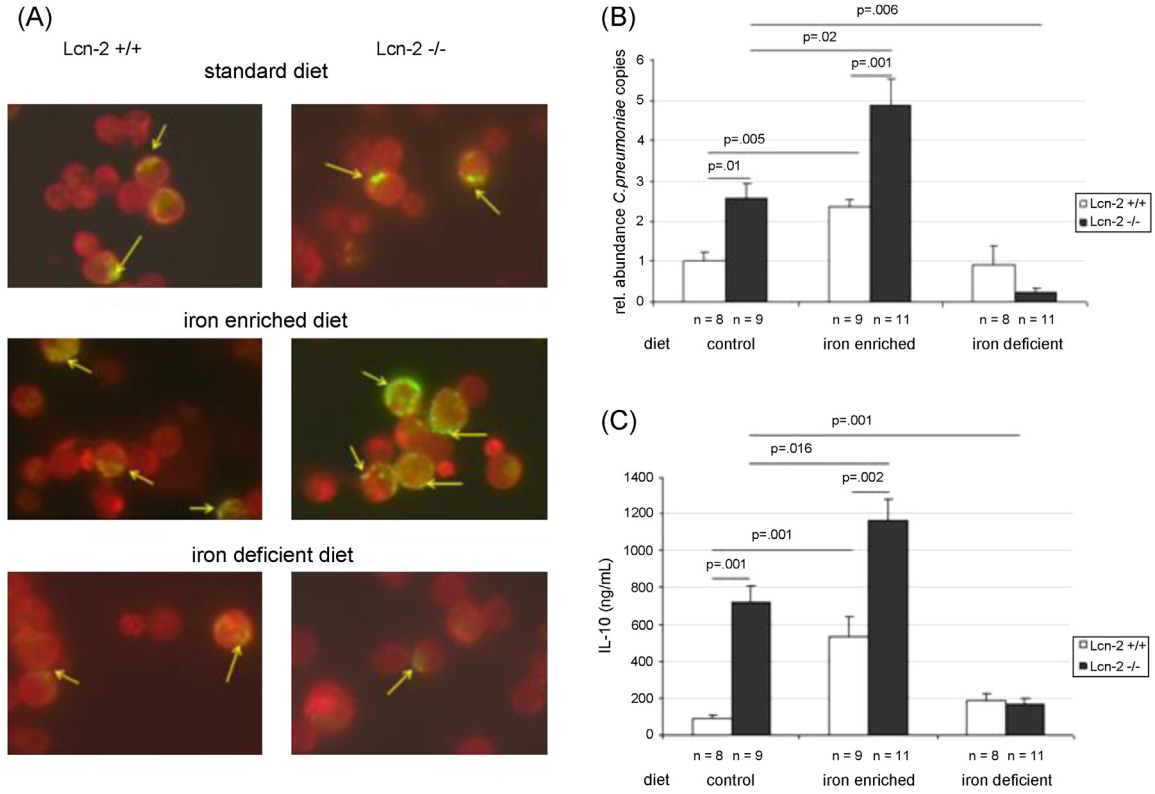


**Fig. 6.** Effect of dietary iron content on the course of *C. pneumoniae* infection and IL-10 formation in the peritoneal cavity. (A and B) Lcn-2 −/− and wt mice were fed an iron enriched, an iron deficient or a standard diet for three weeks followed by intraperitoneal infection with *C. pneumoniae*. After 9–12 h mice were sacrificed and peritoneal macrophages were isolated. The infection with *Chlamydia* was visualized by immunofluorescence and quantified by RT-PCR. Representative slides are depicted at a 500× magnification using a Scope A1 microscope (Zeiss), fluorescein isothiocyanate-marked *C. pneumoniae* inclusions are indicated by arrows (A). Expression of *C. pneumoniae* copies are depicted as means ± SEM (B). (C) In parallel the peritoneal fluid of the three dietary groups of both genotypes was analyzed for IL-10 concentrations by means of ELISA. *p*-Values < 0.05 indicate significant differences between the groups. Numbers (*n*) of animals per group are depicted below.

iron availability because iron supplementation resulted in comparable ferroportin expression in wt and Lcn-2 −/− macrophages. In addition, we observed increased expression of the iron importer DMT-1 in infected Lcn-2 −/− macrophages which likewise aggravates intracellular iron accumulation and the availability of this nutrient for *Chlamydia* thereby resulting in increased proliferation and survival of these pathogens within macrophages (Paradkar et al. 2008; Pan et al. 2010).

Being aware of the fact that intracellular iron levels affect innate immune effector pathways (Wessling-Resnick 2010; Weiss et al. 1992; Weiss 2002; Oexle et al. 2003) we studied the expression of cytokines which have been previously found to be induced by *Chlamydial* infection (Rizzo et al. 2011; Yilma et al. 2012). Thereby, we observed increased IL-10 expression in infected Lcn-2 −/− peritoneal macrophages which would be in a line with increased intracellular iron levels in Lcn-2 −/− macrophages (Devireddy et al. 2005; Nairz et al. 2009) because macrophage iron retention has been shown to be directly associated with increased IL-10 expression (Fritsche et al. 2008). Most surprisingly, aIL-10 treatment deteriorated *Chlamydial* infection indicating that IL-10 expression may be protective in this type of infection (Yilma et al. 2012). This could be proven by stimulation of *C. pneumoniae* infected peritoneal macrophages with IL-10. When studying the underlying mechanism we found that aIL-10 treatment reduced ferritin protein levels in cells which subsequently result in reduced iron storage and increased availability of labile iron in the cell (Esposito et al. 2003). This appears to be cause effective because IL-10 has previously been shown to increase ferritin expression and cellular iron storage (Tilg et al. 2002). Interestingly, while aIL-10 reduced ferritin protein levels in Lcn-2 −/− macrophages it increased ferroportin mRNA levels which could result in induction of iron export. However, in parallel aIL-10 significantly increased hepcidin mRNA expression which has previously been shown to block iron export by degrading ferroportin on the cell surface membrane of macrophages in an autocrine fashion (Theurl et al. 2008). Thus, the increased expression of IL-10 in Lcn-2 −/− macrophages can be considered as a compensatory mechanism to outbalance the high intracellular iron levels by promoting iron storage via incorporating the metal into ferritin rendering this nutrient less available for bacteria.

Moreover, we could confirm these observations by applying an *in vivo* infection model demonstrating that dietary iron challenge affects the course of *C. pneumoniae* infection and IL-10 formation at the site of infection, and that all these events are significantly affected by the presence/absence of Lcn-2. Importantly, the enhanced IL-10 formation and poor control of *C. pneumoniae* infection in Lcn-2 −/− mice could be reversed in mice fed an iron deficient diet, thus pointing to the importance of iron availability and Lcn-2 functionality for the control of *C. pneumoniae* infection. As all, iron overload, macrophage polarization, local cytokine formation and *C. pneumoniae* have been implicated to play decisive roles for the pathogenesis of atherosclerosis (Gaenzer et al. 2002; Campbell and Kuo 2004; Belland et al. 2004; Sullivan 2009; Recalcati et al. 2010; Rosenfeld and Campbell 2011; Pello et al. 2011) it remains to be seen in future investigations whether these pathogenic factors are causally related.

In summary our investigations provide novel information in showing that Lcn-2 plays important roles in host defence against infection with *C. pneumoniae* by modulating macrophage iron homeostasis and that IL-10 exerts a protective role by modulating iron storage within ferritin thereby limiting the availability of this growth factor to the pathogen.

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